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Antibodies to Discontinuous or Conformationally Sensitive Epitopes on the gp120 Glycoprotein of Human Immunodeficiency Virus Type 1 Are Highly Prevalent in Sera of Infected Humans

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We have used an indirect-capture enzyme-linked immunosorbent assay to quantitate the reactivity of sera from human immunodeficiency virus type 1 (HIV-1)-infected humans with native recombinant gp120 (HIV-1 IIIB or SF-2) or with the gp120 molecule (IIIB or SF-2) denatured by being boiled in the presence of dithiothreitol with or without sodium dodecyl sulfate. Denaturation of IIIB gp120 reduced the titers of sera from randomly selected donors by at least 100-fold, suggesting that the majority of cross-reactive anti-gp120 antibodies present are directed against discontinuous or otherwise conformationally sensitive epitopes. When SF-2 gp120 was used, four of eight serum samples reacted significantly with the denatured protein, albeit with ca. 3- to 50-fold reductions in titer. Only those sera reacting with denatured SF-2 gp120 bound significantly to solid-phase-adsorbed SF-2 V3 loop peptide, and none bound to IIIB V3 loop peptide. Almost all antibody binding to reduced SF-2 gp120 was blocked by preincubation with the SF-2 V3 loop peptide, as was about 50% of the binding to native SF-2 gp120. When sera from a laboratory worker or a chimpanzee infected with IIIB were tested, the pattern of reactivity was reversed, i.e., there was significant binding to reduced IIIB gp120, but not to reduced SF-2 gp120. Binding of these sera to reduced IIIB gp120 was 1 to 10% that to native IIIB gp120 and was substantially decreased by preincubation with IIIB (but not SF-2) V3 loop peptide. To analyze which discontinuous or conformational epitopes were predominant in HIV-1-positive sera, we prebound monoclonal antibodies (MAbs) to IIIB gp120 and then added alkaline phosphatase-labelled HIV-1-positive sera. MAbs (such as 15e) that recognize discontinuous epitopes and compete directly with CD4 reduced HIV-1-positive sera binding by about 50%, whereas neutralizing MAbs to the C4, V2, and V3 domains of gp120 were either not inhibitory or only weakly so. Thus, antibodies to the discontinuous CD4-binding site on gp120 are prevalent in HIV-1-positive sera, antibodies to linear epitopes are less common, most of the antibodies to linear epitopes are directed against the V3 region, and most cross-reactive antibodies are directed against discontinuous epitopes, including regions involved in CD4 binding.

The envelope glycoproteins gp120 and gp41 are among the principal targets for the human humoral immune response to infection by human immunodeficiency virus type 1 (HIV-1) (2, 67). All or virtually all neutralizing activity in the sera of HIV-1-infected humans is directed against these proteins and particularly against the external glycoprotein gp120 (65, 87, 88; for reviews, see references 47, 49, and 78). gp120 is a heavily glycosylated protein with a complex domain structure delineated by nine disulfide bonds (39, 41, 77). Some antibodies to gp120 can be detected by analytical techniques that measure binding to denatured protein or to peptide fragments thereof (2, 10, 27, 56, 62, 66, 69, 70). Such antibodies are likely to recognize linear epitopes that are relatively insensitive to protein conformation. A subset of neutralizing polyclonal or monoclonal antibodies (MAbs) to the V3 region of gp120 can be detected under these conditions, implying that this neutralizing epitope is at least partially independent of native structure (20, 29, 40, 51, 58, 62, 69). Other linear epitopes in the V2 and C4 domains can also evoke neutralizing responses, albeit generally to a lesser degree than the V3 region (11, 18, 80). The binding of other neutralizing polyclonal antibodies or MAbs to gp120 is lost when the protein is denatured by exposure to detergents, such as sodium dodecyl sulfate (SDS), and/or by reduction of disulfide bonds (11, 21, 22, 25, 26, 30, 31, 60, 78, 79, 84). The epitopes for such antibodies are therefore discontinuous in that they contain amino acids that are not contiguous in the linear sequence but which are brought into proximity when the protein is folded correctly (38). Alternatively, their epitopes may be linear but sensitive to the overall protein conformation. Examples of MAbs to discontinuous epitopes include the human MAbs 15e (26) and F-105 (60), whose epitopes overlap the complex binding site on gp120 for CD4 (55, 81, 82), the primary cell surface receptor for HIV-1 (72). Other human, murine, and rat MAbs with similar (11, 31, 84) or distinct (14, 25, 68) discontinuous epitopes have also been identified.

Although it has been clear for some time that anti-gp120 antibodies can be directed against both linear and discontinuous epitopes, the relative abundance of such antibodies in human HIV-1-positive sera has not been determined. Here we show that antibodies to discontinuous or conformationally sensitive epitopes are highly prevalent and that the only linear epitope relevant to viral neutralization which is recognized by a major fraction of cross-reactive HIV-1-positive serum antibodies is in the V3 region. About half of the anti-gp120 antibodies present in a pool of three HIV-1-positive serum samples are blocked from binding to gp120 by

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MAbs that overlap the discontinuous CD4-binding site. Thus, the preservation of native conformation in gp120 proteins considered immunogens for vaccine trials is crucial for induction of cross-reactive antibodies capable of binding to native gp120 (21, 22, 30, 34). Furthermore, several HIV-1-positive sera from randomly selected American individuals did not react with the V3 region of gp120 from the IIIB isolate but reacted with that region of SF-2 gp120. Thus, SF-2 or related isolates, such as MN, should have priority for vaccine studies (6, 22).

MATERIALS AND METHODS

Reagents. Recombinant gp120 cloned from HIV-1 IIIB (BH10 clone) was expressed in Chinese hamster ovary (CHO) cells by Celltech Ltd. (Slough, United Kingdom) and obtained from the United Kingdom Medical Research Council AIDS Directed Programme reagent repository (45). Recombinant gp120 cloned from SF-2 was expressed in CHO cells by Chiron Inc. (San Francisco, Calif.) and was a gift from Nancy Haigwood and Kathelyn Steimer (22). gp120 proteins purified from the supernatants of IIIB- or MN-infected H9 cells were obtained from Larry Arthur (National Cancer Institute, Frederick, Md.).

Serum samples 47 to 56 were randomly selected from anonymous HIV-1-positive donors who were patients of New York City hospitals. No particular clinical criteria determined the selection of sera for this study. Serum sample FF3346 from a laboratory worker accidentally infected by the IIIB strain of HIV-1 (laboratory worker serum sample) (89) and serum sample 8/25/87 (chimp serum sample) from a chimpanzee experimentally infected with the same virus (4) were obtained from Peter Nara and William Blattner (National Cancer Institute). The laboratory worker serum sample was taken approximately 7 years after seroconversion, the chimp serum sample was taken 10 weeks after infection. Sera QC2, QC5, and QC6 were from HIV-infected Londoners (42). A pool of these three serum samples (1:1:1) was prepared, and then the purified immunoglobulin (Ig) fraction was directly conjugated to alkaline phosphatase by David Bates and James Fuller (Dako Diagnostics, Cambridge, United Kingdom) to make QC256-AP.

MAbs were obtained from several sources including the AIDS Directed Programme and National Institute of Allergy and Infectious Diseases reagent programs. The MAbs and their donors were as follows: 15e, 21h, F-91, 17-B, and 4.8d, Jim Robinson (26, 68); ICR 39.13g and ICR 39.3b, Jackie Cordell (11); BAT-123, BAT-085, G3-4, G3-136, G3-536, and G3-519, Michael Fung (18, 80); 268-D and 588-D, Susan Zolla-Pazner (19, 31, 32) F-105, Marshall Posner (60); 1661.01, Jon Laman (36); CRA-1, -3, -4, -5, and -6, Mark Page; 178.1.1, 339.1.1, 37.1, 136.1, 213.1, and 187.1, Claudine Brück (83); 221, Rod Daniels; 4G7E5, 2810B1, and 4A7C6, Richard Tedder; 9301, Paul Durda; and C4, C12, C13, B13, and B15, George Lewis (1).

Denaturation of gp120. IIIB gp120 (250 ng/ml) or SF-2 gp120 (5 μg/ml) in Tris-buffered saline (TBS) containing 10% fetal calf serum (TBS-FCS) was mixed with 50 mM dithiothreitol (DTT) with or without 1% SDS and boiled for 5 min. Nine volumes of TBS-FCS containing 1% Nonidet P-40 (TBS–NP-40) were then added, and the solution was cooled to room temperature. Denatured serum proteins were visible as a precipitate in the samples heated with DTT and without SDS, but these proteins were substantially redissolved upon the addition of NP-40.

Binding of sera to native or denatured gp120. Native gp120 diluted in TBS-FCS (25 ng/ml for IIIB; 500 ng/ml for SF-2), or DTT- or SDS-DTT-treated gp120, was captured onto solid-phase-adsorbed sheep antibody D7324 by incubation for 2 h at room temperature (42, 44, 48). This antibody was raised against the carboxy-terminal 15 amino acids of gp120 (LAV-1), which are identical in IIIB and SF-2. The concentrations of gp120 used were chosen to ensure saturation of the solid-phase capture antibody. Unbound gp120 was washed away with TBS, and then HIV-1-positive sera diluted in TBS containing 2% nonfat milk powder (Cadbury's Marvel) and 20% sheep serum (TMSS buffer) was added. For reaction of sera with denatured gp120, either 0.5% Tween 20 or 1% NP-40 was added to the TMSS buffer to ensure that detergent was always present; either detergent was effective, but lower nonspecific serum absorption to the solid phase occurred with Tween 20 than with NP-40, so the former was generally used in experiments when it was desirable to reduce the assay background level to an absolute minimum. At least some of the background absorption of sera to the solid phase in the absence of gp120 was due to antibody reactivity with the sheep capture antibody D7324, and a portion of this may be antiidiotypic in nature. Background absorption in the assay was significant (optical density at 492 nm [OD_{492}] > 0.100) at serum dilutions of <1/3,000, although the extent of background absorption varied for the different serum samples (see Results). Bound HIV-1-positive human or chimpanzee serum antibodies or human MAbs were detected with alkaline phosphatase-conjugated goat anti-human IgG (Accurate Chemicals) diluted 1:4,000 in TMSS buffer. Bound murine MAbs were detected with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Dako Diagnostics) diluted 1:3,000 in TMSS buffer. The AMPAK system (Dako Diagnostics) was used to detect alkaline phosphatase (44).

Binding of sera or MAbs to solid-phase peptides. Peptides CNTRKRIRIQRGPGRAFVTGK and CNTRKSIYIGPGRA FHTTGR from the V3 regions of IIIB (HxB2) and SF-2 gp120 were obtained from American Biotechnologies Inc. (Cambridge, Mass.) and were adsorbed onto enzyme-linked immunosorbent assay (ELISA) wells (Immulon II; Dynatech) by overnight incubation at 1 μg/ml in 100 μl of TBS. MAbs or sera in TMSS buffer were reacted with the peptides for 1 h, and bound antibody was detected as outlined above.

Inhibition of serum antibody binding to gp120 by peptides or MAbs. For competition between V3 peptides and gp120 for serum antibodies, sera were incubated with peptides for 30 min in TMSS buffer without or with 0.5% Tween 20 before being added D7324-immobilized native or denatured gp120. Bound serum antibodies were detected as described above. For competition between MAbs and QC256-AP for gp120, MAbs were incubated with D7324-captured native gp120 at appropriate dilutions in 50-μl portions of TMSS buffer. After 30 min, QC256-AP was added in 50 μl of TMSS buffer at a concentration of approximately 5 μg of human Ig per ml and the incubation continued for another hour before determination of bound QC256-AP. In both cases, background antibody absorption (no gp120, 0% binding value) was subtracted from each OD_{492} value and then the amount of antibody bound at each peptide or MAb concentration was expressed as a percentage of that bound in the absence of peptide or MAb (100% binding). Each data point shows the mean ± standard deviation of triplicate values.

Binding of sCD4 and MAbs to virions. Supernatant HIV-1 RF virions (100 μl) were incubated with recombinant, soluble CD4 (sCD4) (12) for 1 h at 4°C before the addition of a MAb and an additional 1-h incubation at 4°C. The reaction

mixtures were fractionated by gel filtration on Sephacryl S-1000 and the virion fraction was collected as previously described (46). Virions were disrupted by the addition of 1% NP-40 and 10% sheep serum, and then the released gp120-sCD4 or gp120-MAb complexes were captured onto the solid phase by adsorbed antibody D7324. Bound sCD4 was detected with rabbit anti-sCD4 serum CBL-34 and alkaline phosphatase-conjugated sheep anti-rabbit Ig (Dako Diagnostics) (46). Bound MAb was detected with alkaline phosphatase-conjugated goat anti-rat IgG (Accurate Chemicals).

RESULTS

A minority of HIV-1-positive serum antibodies bind denatured gp120. To evaluate the relative quantities of antibodies recognizing discontinuous and linear epitopes on gp120, we used recombinant gp120 from IIIB or SF-2 in an ELISA. In this assay, gp120 was diluted in TBS-FCS (defined here as native gp120) or was denatured by being boiled for 5 min in the presence of 50 mM DTT with or without 1% SDS, followed by a 10-fold dilution into buffer containing 1% NP-40, a nonionic detergent. The latter procedure reduces the SDS concentration both by dilution and by the formation of mixed SDS–NP-40 micelles and in addition helps to maintain gp120 in a denatured state. The native or denatured gp120 was then captured onto the solid phase by means of an immobilized sheep polyclonal antibody to a peptide from the conserved gp120 carboxy terminus. This antibody is reactive with both native and denatured recombinant gp120 (IIIB and SF-2) and can detect gp120 in a variety of assays, including ELISA, Western blotting (immunoblotting), and radioimmunoprecipitation assay (42, 44, 48). Native recombinant or virus-derived gp120 captured onto the solid phase via its carboxy terminus reacts with sCD4 and MAbs to discontinuous epitopes with high affinity, indicating that immobilizating gp120 in this way does not have a significant effect on the structure of the molecule (26, 42). It may be noted that the carboxyl-terminal region of gp120 is implicated in gp41 contact (23, 73).

First, we confirmed that boiling gp120 with DTT with or without SDS destroyed the ability of gp120 to react with sCD4 or MAbs that recognize discontinuous epitopes sensitive to the conformation of gp120. A total of 16 human, mouse, and rat MAbs (15e, 21h, ICR 39.13g, ICR 39.3b, BAT-123, BAT-085, G3-4, G3-136, CRA-3, CRA-4, CRA-5, CRA-6, 178.1.1, 339.1.1, 4G7E5, and 2810B1) and sCD4 were found either not to bind to denatured gp120 (IIIB) or to bind very poorly compared with their binding to the native protein (data not shown). Conversely, we identified 14 murine MAbs (37.1, 136.1, 213.1, 221, CRA-1, 187.1, 1661.01, 4A7C6, 9301, C4, C12, C13, B13, and B15) that bound exclusively or preferentially to denatured gp120 (IIIB) in this assay. The increase in titer for denatured gp120 compared with that of native gp120 was >100-fold for some MAbs (data not shown). These experiments confirm that we were able to efficiently capture gp120 onto the solid phase after denaturation and that the presence of SDS did not significantly occlude antibody epitopes under our assay conditions. MAbs CRA-1 and 213.1 were also found to react preferentially with denatured (compared with native) SF-2 gp120 (data not shown). SDS was not usually necessary for exposure of these cryptic regions of gp120, as boiling in the presence of DTT was sufficient. Similarly, the omission of SDS in the denaturing step did not significantly affect human serum titrations against denatured gp120 (see below), in that

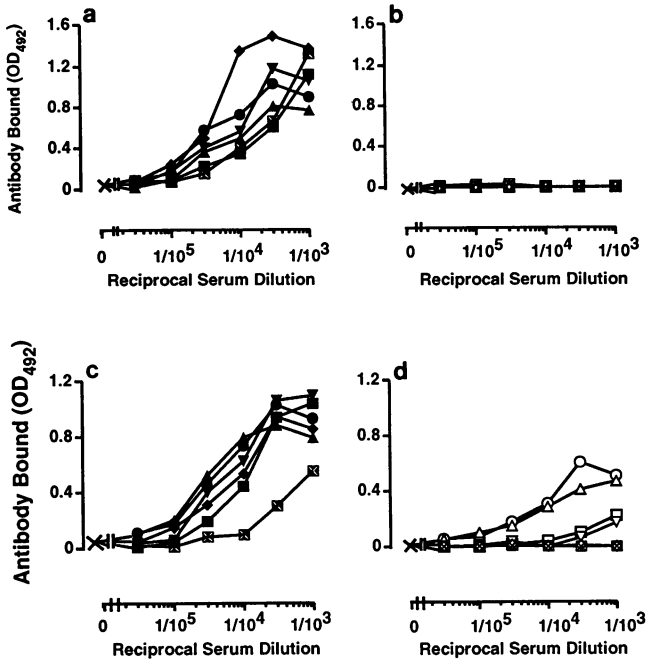

FIG. 1. Reactivities of HIV-1-positive human sera with native or denatured recombinant IIIB or SF-2 gp120. Recombinant IIIB (a and b) or SF-2 (c and d) gp120 was captured onto the solid phase either with the protein in the native conformation (a and c; open symbols) or after denaturation by being boiled in the presence of DTT (b and d; closed symbols). HIV-1-positive sera at the dilutions indicated were then reacted with gp120, and bound antibodies were detected. OD_{492} values at each serum dilutions were corrected by subtraction of background absorption due to nonspecific antibody absorption in the absence of gp120. Negative values were scored as zero for ease of plotting and were always less than −0.100 and usually less than −0.050. The assay background absorption in the absence of serum was 0.082 ± 0.011, and with serum dilutions of 1/1,000, it was generally in the range of 0.15 to 0.30, depending on the particular serum sample and whether detergent was present. Open symbols not visible are obscured beneath the visible open symbol. Symbols: ● and ○, serum sample 49; ■ and □, serum sample 51; ▲ and △, serum sample 52; ▼ and ▽, serum sample 54; ◆ and ◇, serum sample 55; ⊠ and ⊠ , serum sample 56; ×, no serum.

the titration curves were similar within approximately twofold; therefore, SDS was generally omitted.

To determine the reactivities of HIV-1-positive human sera with native and denatured gp120 (IIIB and SF-2), we used a panel of nine serum samples randomly selected from HIV-infected New Yorkers. In a preliminary experiment, five of these serum samples (serum samples 47, 48, 50, 55, and 56) were found not to react significantly with SDS-DTT-treated IIIB or SF-2 gp120 (see below): three of these samples (serum samples 47, 48, and 50) were not studied further. The remaining six serum samples were titrated against native IIIB and SF-2 gp120 and against the same proteins denatured by being boiled in the presence of DTT, but without SDS. Each of the six serum samples bound with a high titer to both native proteins (Fig. 1a and c). However, none of the six serum samples reacted significantly with denatured IIIB gp120 at concentrations below 1/1,000 (Fig. 1b), and two of the six (serum samples 55 and 56) did not react with denatured SF-2 gp120 (Fig. 1d). Of the four serum samples that did recognize denatured SF-2 gp120, the reac-

tivities of two (serum samples 51 and 54) were weak, and they were not evaluated further. However, two serum samples (serum samples 49 and 52), reacted reasonably well with denatured SF-2 gp120 (Fig. 1d). The titration curves for these sera against denatured SF-2 gp120 were shifted to the right by approximately 10-fold compared with the titration curves for sera against the native glycoprotein. Thus, about 10 to 15% of the total anti-gp120 antibodies in these sera reacted with linear epitopes available on denatured SF-2 gp120, but not on denatured IIIB gp120. This conclusion is not critically affected by the magnitude of background serum absorption in the absence of gp120, which varies between sera; even if no allowance is made for background absorption and it is assumed that all ELISA reactivity with the wells containing denatured gp120 is due to specific binding, the highest signals obtained against denatured IIIB gp120 with any serum sample (serum sample 55) at a dilution of 1/1,000 corresponded to <1% of its reactivity with native IIIB gp120.

The individuals whose sera were tested are extremely unlikely to have been infected by an isolate closely resembling IIIB. SF-2, like MN, is more likely to be representative of viruses circulating in the American population (13, 37, 90). We reasoned, therefore, that the failure of sera from our panel to react with denatured IIIB gp120 could be due to variation in linear epitope sequences between the IIIB virus and those more prevalent in the general population. To evaluate this, we tested sera from a laboratory worker accidentally infected with the IIIB isolate (89) and from a chimpanzee experimentally infected with the same virus (4) (Fig. 2). The laboratory worker serum sample reacted with a high titer against native IIIB gp120, but its reactivity with native SF-2 gp120 was about 10-fold lower (Fig. 2a) (43). Denaturation of IIIB gp120 reduced the binding of the laboratory worker serum sample by about 10-fold compared with that of native IIIB gp120, and there was no significant reactivity with denatured SF-2 gp120, indicating that there was at least a 100-fold loss of titer compared with that against native SF-gp120. A similar pattern of responses was obtained with the IIIB-infected chimp serum sample (Fig. 2b), although the overall titers of the sera against both native proteins were at least fivefold less than those of the laboratory worker serum sample. There was no significant reactivity of the chimp serum sample with denatured IIIB or SF-2 gp120, although it was notable that the assay background levels with the chimp serum sample were significantly greater than those with any of the other sera tested, and a weak reaction with denatured IIIB gp120 might be obscured by this phenomenon. The above data are summarized for clarity in Table 1. In experiments of similar design to those described above (Fig. 1 and 2), we obtained similar results when we used IIIB virus-derived gp120 (in place of recombinant IIIB gp120) and MN virus-derived gp120 (instead of recombinant SF-2 gp120) (data not shown). Thus, our results do not depend on the particular gp120 preparation used, provided that the protein has the native configuration.

The major epitope for HIV-1-positive sera on denatured gp120 is the V3 loop. The laboratory worker and chimp serum samples were clearly distinguishable from the random panel of HIV-1-positive serum samples in their reactivities with native and denatured IIIB and SF-2 gp120. The pattern of responses (i.e., random HIV-1-positive sera showing higher reactivities with denatured SF-2 gp120 but both laboratory worker and chimp serum samples showing higher reactivities with denatured IIIB-gp120) suggested that a major fraction of the linear epitopes recognized by the sera

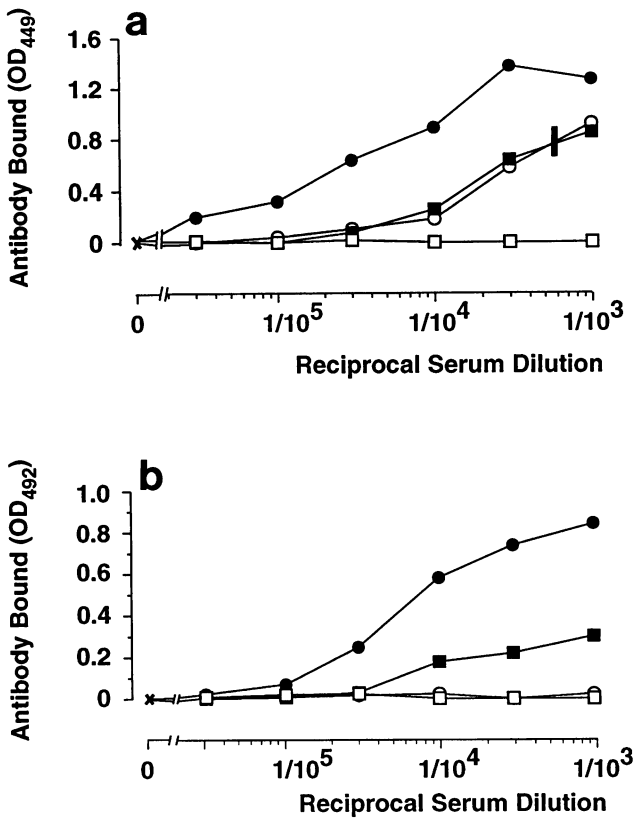

FIG. 2. Reactivities of sera from an HIV-1 IIIB-infected laboratory worker or chimpanzee with native or denatured IIIB or SF-2 gp120. Recombinant IIIB (● and ○) or SF-2 (■ and □) gp120 was captured onto the solid phase either with the protein in the native conformation (closed symbols) or after denaturation by being boiled in the presence of DTT (open symbols). Sera from a HIV-1 IIIB-infected laboratory worker (a) or a chimpanzee (b) at the concentrations indicated were then reacted with gp120, and bound antibodies were detected. OD_{492} values were corrected for background levels, as described in the legend to Fig. 1. The assay background absorption in the absence of serum was 0.054 ± 0.007. At serum dilutions of 1/1,000, background absorption levels were 0.075 and 0.112 for the laboratory worker serum sample and 0.124 and 0.443 for the chimp serum sample under the conditions of the native and denatured gp120 assays, respectively. ×, no serum.

was likely to be type specific. The obvious candidate for such an epitope was the V3 region of gp120, a relatively type-specific, partially linear neutralizing domain known have an unusual sequence in the IIIB isolate. We therefore measured the binding of our serum panels to solid-phase-absorbed 21-mer peptides from the V3 regions of IIIB and SF-2 gp120. Of the five tested serum samples (serum samples 49, 50, 52, 54, and 55) from the random HIV-1-positive serum panel, only serum samples 49 and 52 reacted significantly at 1/1,000 dilution with SF-2 peptide in the absence of detergent (OD_{492} = 0.26 and 0.13). There was a very weak reaction of the other three serum samples with SF-2 peptide (OD_{492} = 0.04 to 0.05 above background absorption). None of the five serum samples reacted at all with the IIIB V3 peptide (OD_{492} < 0.01 above background absorption). It was notable that the two serum samples reacting best with the SF-2 peptide (serum samples 49 and 52) were also the most reactive with denatured SF-2 gp120, with serum sample 49 consistently exhibiting more binding than serum sample 52 in

TABLE 1. Summary of reactivities of HIV-1-positive sera with gp120 or V3 peptides

Serum sample	Reactivity[a] of the serum sample with the following peptide:					
	IIIB gp120		SF-2 gp120		IIIB V3 peptide (solution or solid phase)	SF-2 V3 peptide (solution or solid phase)
	Native	Denatured	Native	Denatured		
47	++	−	++	−	ND	ND
48	++	−	++	−	ND	ND
49	++++	−	++++	++	−	++
50	++	−	++	−	−	+/−
51	++++	−	++++	+	ND	ND
52	++++	−	++++	++	−	++
54	++++	−	++++	+	−	+/−
55	++++	−	++++	−	−	+/−
56	++++	−	++++	−	ND	ND
QC256-AP	++++	−	++++	++	−	++
Laboratory worker	++++	++	++	−	++	−
Chimp	+++	−	+	−	+	−

[a] Data were derived from the experiments described in the legends to Fig. 1 to 4 and the text. The numbers of plus signs by each serum sample are approximately proportional to its reactivity with the antigen indicated. Those symbols representing reactivity with gp120 proteins are quantitatively comparable with each other, but not with the V3 peptide reactivities. ND, not done; −, not reactive; +/−, very weak reactivity.

both assays. Conversely, neither the laboratory worker serum sample nor the chimp serum sample bound significantly to the SF-2 V3 peptide in the absence of detergent, but each recognized the IIIB V3 peptide, with OD_{492} values at 1/1,000 and 1/300 dilutions of 0.49 and 1.17, respectively, for the laboratory worker serum sample and 0.17 and 0.38, respectively, for the chimp serum sample. Thus, the pattern of reactivity of the laboratory worker serum sample with V3 peptides was also consistent with that against the denatured gp120 proteins (Table 1). A detailed comparison of the reactivities of these and other HIV-1-positive sera with solid-phase V3 peptides and the V3 loop on native gp120 will be described elsewhere (43).

We determined the relative contribution of the V3 loop to the epitopes recognized by HIV-1-positive sera on denatured gp120 by performing solution-phase competition experiments in which sera were incubated with V3 peptides before denatured gp120 was added. In a preliminary experiment, we determined that each of the four serum samples (serum samples 49, 51, 52, and 54) capable of reactivity with SDS-DTT-denatured SF-2 gp120 failed to react after preincubation with SF-2, but not IIIB, V3 loop peptide at concentrations from 0.01 to 1 μg/ml. This was evaluated in more detail with the two most reactive serum samples (serum samples 49 and 52). The binding of these sera to denatured SF-2 gp120 was completely inhibited by the SF-2 V3 loop peptide, while the corresponding IIIB peptide had no effect (Fig. 3). Conversely, the binding of the laboratory worker serum sample to denatured IIIB gp120 was inhibited by the IIIB, but not by the SF-2, V3 loop peptide (Fig. 3). A small proportion (about 25%) of the reactivity of the laboratory worker serum sample with denatured IIIB gp120 was resistant to inhibition by V3 peptide, suggesting that other linear epitopes can account for a very small fraction of the total anti-gp120 activity in this serum sample. Since antibodies binding to denatured gp120 account for about 10% of the total (see above), we estimate that antibodies to this linear, non-V3, epitope(s) account for no more than 3% of the total anti-gp120 reactivity, and it is possible that these antibodies do not react with native gp120.

The V3 loop binds a significant fraction of antibodies reactive with native gp120. The V3 loop of gp120 is capable of being a linear epitope in that antibodies raised against peptides will react with native gp120 and neutralize the virus, and human HIV-1-positive sera will react with appropriate V3 loop peptides (47, 49). However, a significant fraction of anti-V3 loop antibodies might recognize conformationally sensitive epitopes and hence not react with denatured gp120 (50). To investigate this possibility, we tested whether V3 loop peptides could block binding of serum antibodies to native gp120 (IIIB or SF-2) (Fig. 4). In initial experiments, the procedure was similar to that used in the experiments described in the legend to Fig. 3, except that the serum-peptide complexes were reacted with native

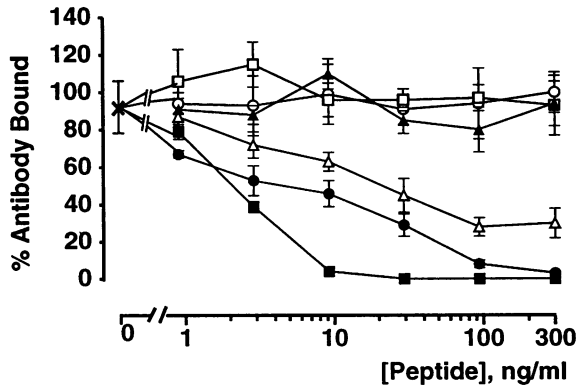

FIG. 3. Effect of V3 loop peptides on the binding of HIV-1-positive sera to denatured IIIB or SF-2 gp120. IIIB or SF-2 gp120 was denatured by being boiled in the presence of DTT and then captured onto the solid phase. HIV-1-positive sera at a fixed concentration were incubated for 1 h with V3 loop peptides at the concentrations indicated, then the mixture was added to gp120, and bound antibodies were detected. The conditions and 0 and 100% binding values were as follows: serum sample 49 reactivity with SF-2 gp120 plus SF-2 (●) or IIIB (○) V3 loop peptide, with a 0% binding value of 0.198 ± 0.043 and a 100% binding value of 1.000 ± 0.094; serum 52 reactivity with SF-2 gp120 plus SF-2 (■) or IIIB (□) V3 loop peptide, with a 0% binding value of 0.114 ± 0.013 and a 100% binding value of 0.874 ± 0.088; laboratory worker serum reactivity with IIIB gp120 plus SF-2 (▲) or IIIB (△) V3 loop peptide, with a 0% binding value of 0.090 ± 0.018 and a 100% binding value of 0.757 ± 0.071.

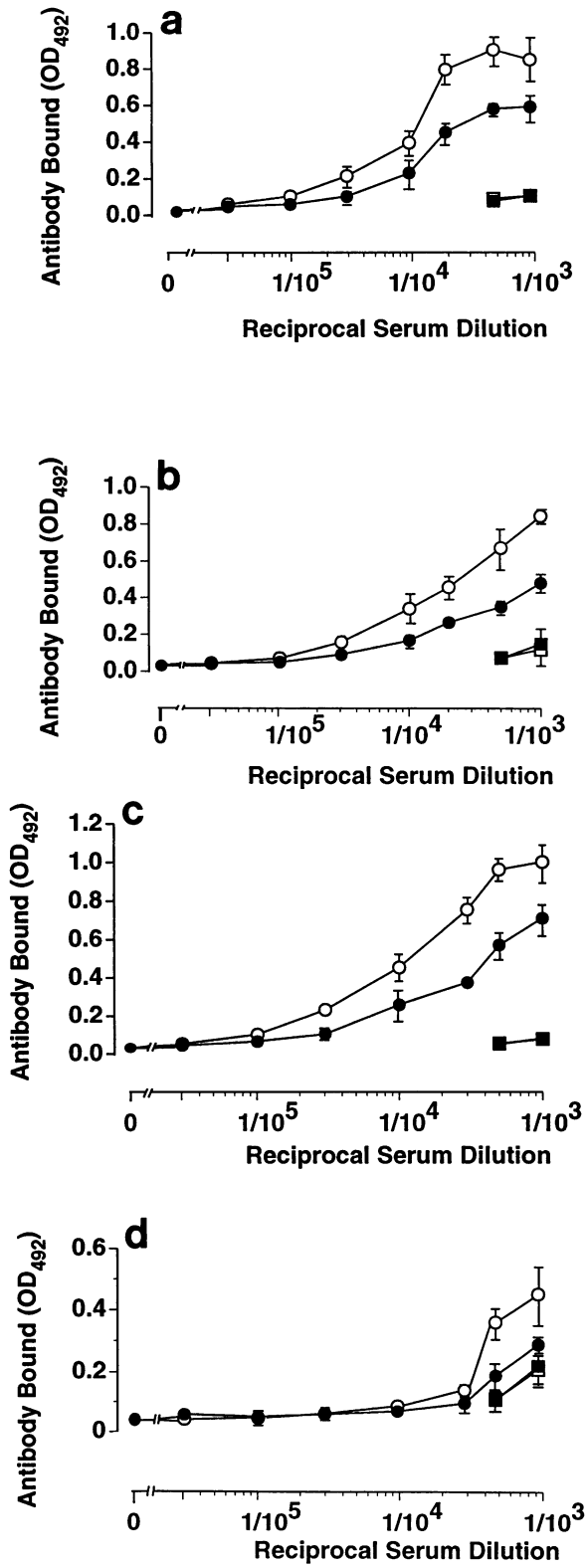

FIG. 4. Effect of V3 loop peptides on the reactivities of HIV-1-positive sera with native IIIB or SF-2 gp120. Native IIIB or SF-2 gp120 was captured onto the solid phase. HIV-1-positive sera at the concentration indicated were incubated for 1 h with (closed symbols) or without (open symbols) the appropriate V3 loop peptide (300 ng/ml) before the addition of gp120 and determination of bound antibody (● and ○). Background serum absorption (no gp120) at the highest serum concentration is also represented (■ and □). (a) Serum sample 49 with SF-2 gp120 with and without SF-2 peptide, (b) serum sample 52 with SF-2 gp120 with and without SF-2 peptide, (c) laboratory worker serum sample with IIIB gp120 with and without IIIB peptide, (d) chimp serum sample with IIIB gp120 with and without IIIB peptide.

gp120. The reactivities of serum samples 49 and 52 (1/2,000 or 1/10,000 dilution) with native SF-2 gp120 were reduced by about 50% by the SF-2 V3 peptide (10 to 300 ng/ml), with the IIIB V3 peptide having no effect. Binding of the laboratory worker serum sample to native IIIB gp120 was inhibited by the IIIB V3 peptide by about 30 and 60% at 1/2,000 and 1/10,000 dilutions, respectively, while the SF-2 peptide was not inhibitory. The chimp serum sample (1/2,000) reactivity with native IIIB gp120 was inhibited by about 65% by the IIIB V3 peptide, but not by the SF-2 peptide. Half-maximal inhibition of serum binding (1/10,000 dilution) was generally observed at about 2 ng of V3 peptide per ml (data not shown). It was notable that the binding of serum samples 49 and 52 to native IIIB gp120 and of the laboratory worker serum sample to native SF-2 gp120 were not inhibited (<10% reduction) by either V3 peptide (data not shown). Thus, the cross-reactive antibodies in these sera are not capable of binding to the V3 loops of these proteins, at least when they are represented by peptides. A more comprehensive study of this issue, demonstrating that cross-reactive V3 loop antibodies can sometimes be detected in solution-phase competition assays with native gp120 proteins will be described elsewhere (43).

Titration of serum samples 49 and 52 and the laboratory worker serum sample against native gp120 (IIIB or SF-2) before and after preincubation with an optimally inhibitory concentration of the homologous V3 loop peptide indicated that the binding curves were generally shifted to the right by about two- to threefold in the presence of the V3 peptide (Fig. 4). When saturation binding was achieved (serum sample 49), the maximum level of antibody binding was reduced by approximately 40% in the presence of the competing SF-2 V3 peptide (Fig. 4a). Thus, a significant portion of the antibody reactivity of these sera with native gp120 is dependent on the V3 loop.

The CD4-binding site of gp120 is a major target for HIV-1-positive serum antibodies. In addition to the V3 loop, several other neutralizing epitopes are present in gp120. These neutralizing epitopes include regions in the V2 and C4 domains which can function at least in part as linear epitopes (18, 80). The principal discontinuous neutralizing epitopes on gp120 overlap with the CD4-binding site (55, 81, 82), although there are other, less potent, discontinuous sites that either do not overlap or only partially overlap with that for CD4 (25, 68). Most of these epitopes are recognized by HIV-1-positive human serum antibodies in that MAbs can be blocked from binding to gp120 by preincubation with human serum, as can sCD4 (18, 25, 26, 42). Such assays do not, however, determine how prevalent antibodies to discontinuous epitopes are in HIV-1-positive sera. To address this issue, we performed a reverse inhibition experiment in which we prebound MAbs onto native gp120 and then measured the binding of alkaline phosphatase-conjugated anti-HIV Ig. (The properties of these MAbs are summarized in Table 2.) Because the sera used in the experiments shown in Fig. 1 to 4 were not available in sufficient quantity to allow

TABLE 2. Properties of MAbs tested for blocking HIV-1-positive serum binding to gp120[a]

MAb	gp120 epitope	Neutralization of IIIB (reference)	Reactivity with SF-2 gp120	Inhibition of HIV-1 sera binding to IIIB gp120
15e	Discontinuous, overlapping CD4-binding site	++ (26)	+	++
21h	Discontinuous, overlapping CD4-binding site	++ (68)	+	++
F-91	Discontinuous, overlapping CD4-binding site	++ (68)	+	++
F-105	Discontinuous, overlapping CD4-binding site	++ (60)	ND	++
588-D	Discontinuous, overlapping CD4-binding site	++ (31)	ND	++
ICR 39.13g	Discontinuous, overlapping CD4-binding site	+ (11)	ND	++
G3-519	C4	+ (80)	+	+/−
G3-536	C4	+ (80)	+	+/−
G3-4	Discontinuous, V2	+ (25)	−	−
G3-136	V2	+ (18)	−	−
BAT-085	V2	+ (18)	−	−
4.8d	Discontinuous	+ (68)	+	−
BAT-123	V3	+++ (18)	−	−

[a] Results are derived from experiments described in the text except for neutralization data, which are taken from the original reports cited or from our unpublished results (68). The number of pluses is proportional to the magnitude of the effect. Data are comparable only within a column. ND, not done.

Ig purification and labelling, we used a pool of anti-HIV Ig purified from the serum samples of three donors and then labelled with alkaline phosphatase. We first characterized this serum pool (QC256-AP) in the assays depicted in Fig. 1 to 4. QC256-AP bound to native SF-2 and IIIB gp120 with high titers, but its titer against denatured SF-2 gp120 was about 10-fold lower and there was negligible binding to denatured IIIB gp120 ($\geqslant$ 100-fold reduction in titer compared with that of the native protein). The binding values of QC256-AP to native and denatured SF-2 gp120 were reduced by approximately 25 and 85%, respectively, by optimal concentrations of SF-2, but not IIIB, V3 peptides. Thus, QC256-AP behaves like serum samples 49 and 52 (Table 1).

We confirmed that each of the MAbs tested bound to IIIB gp120 by preparing titration curves and testing the MAb's ability to inhibit sCD4 binding to gp120 (data not shown). Appropriate concentrations of MAbs were then bound to gp120 before the addition of a fixed concentration of QC256-AP to the mixture. Of MAbs BAT-085, G3-136, G3-4, and 4.8d, which bind to the V2 domain or to discontinuous epitopes only partially overlapping the CD4-binding site, none had a significant effect on QC256-AP binding ($\pm$ 20%, the limit of random variation) (Fig. 5). There was no inhibition by BAT-123 directed at the IIIB V3 loop (Fig. 5b). Each of the MAbs overlapping the CD4-binding site that we tested, 15e, 21h, F-91, ICR 39.13g, F-105, and 588-D, significantly inhibited QC256-AP binding by 40 to 60% (Fig. 5 and data not shown). The extent of inhibition was slightly variable between experiments but was typically 50%; none of the MAbs was notably more effective than the others, and each was maximally inhibitory at concentrations that saturated gp120 (data not shown). Two MAbs to the C4 domain (G3-536 and G3-519) were also tested. Each significantly inhibited QC256-AP binding (Fig. 5 and data not shown), but this inhibition was generally weaker than seen with the MAbs that overlap the CD4-binding site and was rather variable between experiments. This variability may be caused by the fact that the extent of inhibition (about 30%) was close to the limit of significance in this type of assay. We attempted a similar competition experiment using MAbs that could be demonstrated to bind to native SF-2 gp120. These MAbs included 15e, 21h, and F-91 (which overlap the CD4-binding site), G3-519 (to the C4 region), 17-B and 4.8d (to discontinuous epitopes), and 268-D (to the V3 loop). None of these MAbs inhibited QC256-AP binding to SF-2 gp120 to a significant extent (>25%), although two of MAbs with the CD4-binding site (15e and F91) caused a degree of inhibition that was close to being significant (data not shown). We attribute this failure to the wider range of epitopes recognized on SF-2 gp120 by this HIV-1-positive serum pool than on IIIB gp120 (43), so that no one MAb can reduce overall binding to a major extent.

sCD4 and a MAb that overlaps the CD4-binding site on gp120 compete for binding to virions. Since antibodies competing with the CD4-binding site of gp120 are so prevalent in HIV-1-positive sera, we tested whether a MAb to this region could compete with sCD4 for binding to virions. Although MAbs of this class have been shown elsewhere to inhibit the interactions of recombinant gp120 with CD4 or sCD4 (11, 26, 60), their effects on cell-free virions have not been described. For technical reasons relating to immunological cross-reactivities in ELISAs, we used a rat MAb, ICR 39.13g (11). Like most other MAbs of this type, ICR 39.13g is weakly neutralizing (11). We also used RF virus, as this virus routinely has the highest titer of common laboratory-adapted strains, and the amount of virion gp120 present is often limiting for other strains (46). Finally, to minimize the complication of sCD4-induced gp120 shedding, we performed the competition experiment at 4°C (46). sCD4 binding to RF virions clearly blocked ICR 39.13g binding to the same particles (Fig. 6). Thus, MAbs of this type probably compete directly with cellular CD4 for the CD4-binding site on gp120, although this competition remains to be proven unambiguously, as does the relevance of this mechanism for viral neutralization.

DISCUSSION

Although we have been able to analyze only one truly autologous gp120-serum sample pair (the IIIB-infected laboratory worker serum sample) and although our competition analyses (Fig. 5) have used only a single pool of three serum samples, there are several conclusions to be drawn from our study that we believe will be generally relevant. First, antibodies to linear epitopes present on denatured gp120 account for only a small proportion (<10%) of the total anti-gp120 activity in the HIV-1-positive sera we have evaluated. In the nonhomologous situation (e.g., SF-2 gp120 and the IIIB-infected laboratory worker serum sample), the

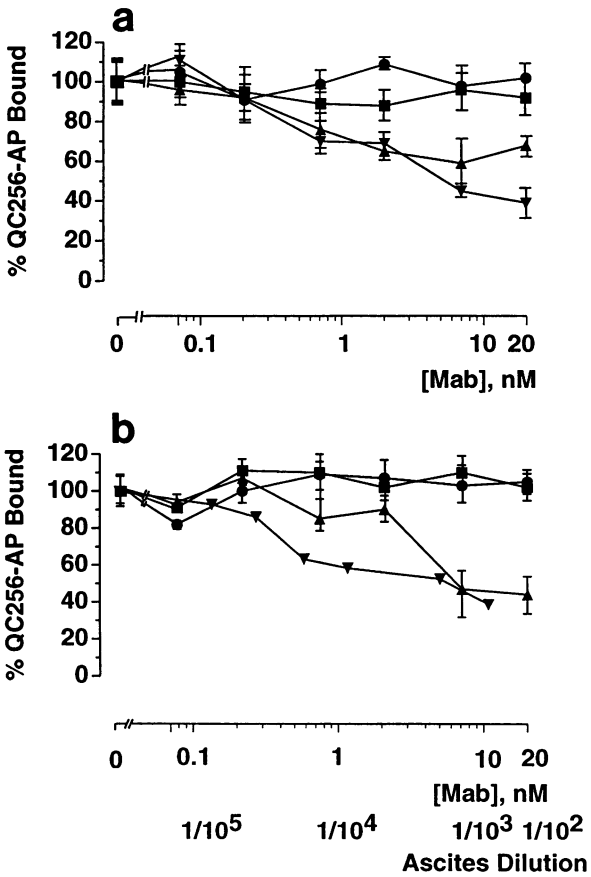

FIG. 5. Competition between anti-gp120 MAbs and HIV-1-positive sera for binding to IIIB gp120. Native IIIB gp120 captured onto the solid phase was reacted for 30 min with MAbs at the concentrations indicated, a fixed concentration of alkaline phosphatase-conjugated HIV-1-positive serum sample (QC256-AP) was then added to the mixture, and the incubation was continued for another hour before determination of bound QC256-AP antibodies. Binding values of 0 and 100% varied between experiments but typically ranged from 0.060 to 0.080 and 0.660 to 0.750, respectively. The MAbs used were as follows: (panel a) BAT-085 (●), G3-136 (■), G3-536 (▲), and 15e (▼); (panel b) BAT-123 (●), G3-4 (■), 21h (▲), and ICR 39.13g (▼). Each MAb was used as purified antibody with concentrations expressed in nanomolar, except for ICR 39.13g, which was ascitic fluid; its concentrations are expressed as dilutions of the ascitic fluid.

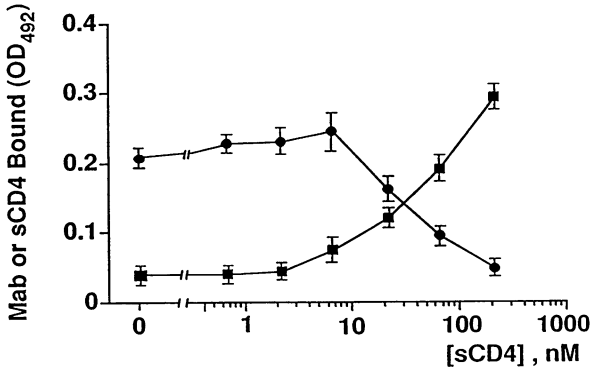

FIG. 6. Competition between a MAb that overlaps the CD4-binding site and sCD4 for binding to RF virions. RF virions were incubated for 1 h at 4°C with sCD4 at the concentrations indicated before the addition of 100 nM ICR 39.13g and another 1-h incubation of the reaction mixture at 4°C. The virions were separated from free sCD4, MAb, and gp120 by gel filtration and disrupted with NP-40 detergent. gp120-MAb (●) and gp120-sCD4 (■) complexes were determined by ELISA.

proportion of antibodies to linear epitopes is even lower (<1%). The principal epitope recognized on denatured gp120 is the V3 loop, which is consistent with the poor reactivity we find in the nonhomologous situation. Other linear epitopes are scarce and may be unimportant for virus neutralization in practice. Thus, analyzing the immune response to gp120 using Western blotting (56) or solid-phase peptide serology (13, 35, 52, 54, 59, 61, 75, 86), however complicated and expensive the method employed (85), yields information on a very minor subset of the total antibody population: we estimate that <1% of the total anti-gp120 antibodies in randomly selected HIV-1-positive sera will bind to short peptides from the IIIB sequence or to denatured gp120 under Western blotting conditions. Conclusions drawn from such studies are of restricted value when the total immune response to gp120 is considered (also see reference 43). It must, however, be noted that our standard procedure of prebinding gp120 onto a carboxy-terminal antibody does mean that HIV-1-positive serum antibodies to this region of gp120 will not detectable in our assays. As the carboxy terminus of gp120 is an immunodominant linear epitope in HIV-1-positive sera, as judged by solid-phase peptide assays (35, 59), our conclusions must be qualified to reflect the absence of this subpopulation of antibodies from our analysis. We do not consider this a serious flaw, as antibodies to this carboxy-terminal epitope of gp120 are not neutralizing (31, 42, 76) and do not bind well to gp120 when present as a gp41 complex, probably because of steric occlusion of the epitope by gp41 (23, 71, 73). It may be noted that another study using Western blotting has concluded that most antibodies from random HIV-1-positive sera that are capable of reacting with denatured IIIB gp120 bind to the V3 loop (56). By inference, the carboxy terminus accounts for a minor proportion of anti-gp120 reactivity with denatured IIIB gp120.

We do not suggest that there is no reactivity at all of our test sera with denatured gp120 (IIIB). High concentrations will probably be reactive in Western blots, although we did not test this. There may also be weak reaction with linear epitopes detectable at high serum concentrations. Higher serum concentrations than a 1/1,000 dilution could not be tested in our ELISA system because of background absorption of antibody to the solid phase independently of gp120. Our conclusion is not that there are no linear epitopes on IIIB gp120 recognized by HIV-1-positive sera but that these epitopes form a very small proportion of the total epitopes available for reaction on the native glycoprotein. How the relative amounts of antibodies to linear and discontinuous epitopes change during the progression of HIV-1 infection will be a subject for further study. The sera we have evaluated so far were not selected on clinical grounds, and longitudinal studies will be necessary to address this issue.

Our second conclusion is that the V3 loop can account for a significant fraction (up to 50%) of the total antibody reactivity with native gp120, provided that the gp120 used is homologous (or approximately so) with the serum sample. Thus, the IIIB V3 loop peptide can compete for laboratory worker serum sample binding to native IIIB gp120, while the

SF-2 peptide cannot. The converse is found with random HIV-1-positive sera, for which the SF-2 (but not IIIB) V3 peptide is an effective competitor with antibodies from some, but not all, of the sera we tested. Although it is clear that broadly reactive V3 loop MAbs exist in HIV-1-positive sera or can be made experimentally (19, 24, 28, 32, 74), those antibodies cross-reacting with heterologous gp120 (random HIV-1-positive sera with IIIB gp120, laboratory worker serum sample with SF-2) were not significantly inhibited from binding by the IIIB or SF-2 V3 peptide. Thus, cross-reactive V3 antibodies may be scarce in those sera we have studied and constitute a very small fraction of the total cross-reactive antibodies. Such antibodies can, however, be detected in some HIV-1-positive sera by solution-phase V3 peptide competition assays (43). It was notable that up to 50% of the reactivity of antibodies with native gp120 could be blocked by solution-phase V3 peptides, whereas <10% of the reactivity in the same sera bound to the V3 loop on denatured gp120. These observations are not paradoxical; many V3 MAbs react significantly less well (10- to 1,000-fold) with denatured gp120 than with the native protein, despite the essentially linear nature of their epitopes (43a). In addition, a proportion of antibodies (monoclonal or otherwise) to the V3 loop may have complex or discontinuous epitopes (50). We also find that several MAbs to linear peptide epitopes in the C2 and C4 domains bind with over 100-fold-reduced affinity to denatured IIIB gp120 compared with the native molecule (43a). Thus, the presentation of many linear epitopes on gp120 is influenced by the overall protein conformation.

The next main conclusion is that antibodies to discontinuous epitopes involved in CD4 binding also account for a major fraction (up to 50%) of HIV-1-positive serum anti-gp120 (IIIB) antibodies, although we were able to perform this competition assay only with a single pool of three serum samples and there was significantly less competition when SF-2 gp120 was used instead of IIIB gp120. The sera forming the pool are not, however, particularly unusual in their ability to block sCD4-gp120 binding and one serum sample is actually rather weak (42). Thus, we believe our conclusion is likely to be generally valid. We have mapped 10 human MAbs capable of blocking sCD4 binding to gp120, and the number of different amino acids likely to contribute to their epitopes is fairly limited, although there are several variations in the combinations used by different MAbs (43a, 81, 82). Whether all of the HIV-1-positive serum antibodies that can be blocked from binding to gp120 by, for example, MAb 15e can themselves inhibit sCD4 binding is unknown; the competitive effect may be indirect. Other MAbs to discontinuous epitopes had little or no inhibitory action on HIV-1-positive serum binding, although two MAbs to the C4 domain were weakly antagonistic. Thus, although present in HIV-1-positive sera, these MAbs make up a minor fraction; whether this is an important fraction remains to be evaluated. There may be other discontinuous epitopes we cannot evaluate with our assay system; by using recombinant gp120 we will, of course, not detect antibodies whose binding to gp120 is dependent on the quaternary structure of the envelope and we will not be able to detect anti-gp41 antibodies.

Our conclusion that antibodies to the V3 loop and to epitopes overlapping the CD4-binding site account for a major fraction of the total human immune response to gp120 is consistent with a recent report from Chamat et al. (9), who found that antibodies of these specificities accounted for most of the neutralizing activity in HIV-1-positive sera. In earlier studies, Steimer et al. (79) showed that HIV-1-positive serum antibodies to gp120 conformational epitopes were more broadly cross-neutralizing than those to linear epitopes, and Haigwood et al. (22) demonstrated that this type of antibodies were induced in baboons by native, but not by denatured, gp120. Antibodies that block CD4 binding probably account for the broadly neutralizing fraction that could not be removed from HIV-1-positive sera by V3 loop peptides (61). We can make some estimates of the absolute concentrations present in HIV-1-positive sera of antibodies to the V3 loop and of those that overlap the CD4-binding site, although these estimates will, of necessity, be very approximate. First, high-titer HIV-1-positive sera such as serum sample 52 and the laboratory worker serum sample bind half-maximally to IIIB gp120 in our assays at approximately 1/10,000 dilution (Fig. 1 and 4). In the same assay system, human MAbs such as 15e typically bind half-maximally at about 0.3 nM (0.5 μg/ml) (26 and unpublished data). Since these antibodies are fairly prevalent (Fig. 5), for a first approximation it is reasonable to equate the titration curves for the MAbs and the polyclonal serum to provide a very crude estimate of their concentration in HIV-1-positive sera. Such a value would be 250 μg/ml. Callahan and colleagues (8) have previously calculated that the sCD4 blocking activity of HIV-1-positive sera ranges from 50 to 500 μg/ml, and our estimates are similar for the sera we have evaluated here. Finally, 2 ng of V3 loop peptide (molecular mass of 3,000 Da) per ml can reduce by half the binding to gp120 of a 1/10,000 dilution of HIV-1-positive sera. This experiment allows an estimate of about 1 mg of V3 loop antibodies per ml in sera. Thus, a ballpark figure for the anti-gp120 antibody content of a typical HIV-1-positive serum sample is 0.1 to 1 mg/ml, about half of which may be directed against the V3 loop and much of the rest directed against discontinuous epitopes overlapping the CD4-binding site. This constitutes a significant fraction of the total Ig content of human serum (about 10 mg/ml for IgG), but it may be noted that many HIV-1-positive individuals are hypergammaglobulinemic, and that 20 to 50% of Ig molecules released from cultures of B cells from HIV-1-positive individuals are directed against HIV proteins (3, 57). Given that the concentration in serum of antibodies overlapping the CD4-binding site on gp120 may be so high, it is curious that group-specific neutralizing activity in human serum is generally quite low (88), especially for primary viruses (24a). Perhaps there are properties of these viruses that restrict their susceptibility to neutralization. The ability of HIV-1 to mutate and thereby escape neutralization by antibodies blocking CD4 binding (9, 81) may also be an important factor in disease progression.

The fact that such a high proportion of antibodies to gp120 bind only to native gp120 has implications for trials of prophylactic or therapeutic vaccines, if it is accepted that the natural human immune response to gp120 is important in delaying the pathogenic consequences of HIV-1 infection. To raise an appropriate immune response, the quality of the immunogen must be a paramount consideration (22, 30, 34, 45). It may be difficult to obtain significant clinical benefit from vaccine trials or postexposure immunotherapy studies using denatured gp120 or gp160 (15, 64). The abilities of such antigens to induce group-specific, conformationally sensitive, neutralizing antibodies are probably low (22, 34). Antibodies to epitopes hidden in the interior of native gp120, but exposed on denatured gp160 (1), are likely to be of limited value for humoral immunity, although it has been suggested that the induction of such antibodies may be

important (63). Antibodies to discontinuous epitopes of gp120 can be raised using recombinant gp120, provided that the immunogen has the native conformation (11). Comparative studies with chimpanzees indicate that gp120 is a superior immunogen for vaccine purposes than gp160 is (5). Although there may be neutralization sites in the external domain of this protein (7), evidence for the presence of a potent neutralizing epitope in the internal region of gp41 (17, 33) is contradicted by other reports that MAbs to this region do not have neutralizing activity (16) or even enhance infection (53). The claim that amino acids 735 to 752 of gp41 are the binding sites for a major fraction of the neutralizing activity of HIV-1-positive sera (17) requires urgent reexamination. Finally, the paucity in random HIV-1-positive sera of antibodies reactive with the IIIB V3 loop argues against the continued use of the IIIB isolate in human experiments. Isolates such as SF-2 and MN, being more representative of the consensus V3 sequence in the American population (13, 37, 90) are far more sensible choices for vaccine trials and postexposure therapy. Yet, of nine serum samples tested, only four reacted significantly with denatured SF-2 gp120 or SF-2 V3 peptide, suggesting that high-quality recombinant gp120 from several isolates close to the consensus V3 sequence (6, 22) may be required in combination for a practical vaccine.

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